REMARKS

A. Regarding the Amendments

Responsive to the Examiner's comments regarding the specification, a substitute specification (excluding the claims) is submitted herewith as Exhibit A. Accompanying the substitute specification is a statement pursuant to 37 C.F.R. 1.125(b)(1) that the substitute specification contains no new matter. Further pursuant to 37 C.F.R. 1.125(b)(1), submitted herewith as Exhibit B is a marked up copy of the substitute specification illustrating the changes made.

Responsive to the Examiner's comments regarding priority data, Applicants respectfully submit that the combined Declaration and Power of Attorney (filed July 20, 2001) sets forth the relevant priority data. Accordingly, it is respectfully submitted that Applicants have made a proper priority claim under 35 U.S.C. 119(e). Nonetheless, the substitute specification includes express reference to the prior application data.

By the present communication, claims 1-16 have been cancelled without prejudice and new claims 17-21 have been added. New claims 17-21 are supported by the specification and the original claims and add no new matter. For example, the method recited in new claim 17 is supported throughout the specification (e.g., page, 3, paragraph 0011; page 6, paragraph 0027). Further with respect to claim 17, support for the phrase "ribonucleoprotein comprising an RNA derived from a non-segmented (-)RNA virus" is found in the specification at page 4, paragraph 0020, and page 5, paragraph 0023. Support for this phrase is also found in original claim 4. Also in claim 17, support for the phrase "the ribonucleoprotein has autonomous replication ability" is found at page 5, paragraph 0023. Finally, support for the phrase "the RNA comprises a foreign gene, and lacks a gene encoding Matrix (M) protein or comprises an inactivated gene encoding M protein" is found in original claims 2, 3, 5, and 6. With respect to claim 18, the citations set forth above for claim 17 also support claim 18. Further support for claim 18 is found at page 3, paragraph 0015 and at page 6, paragraph 0027. Claims 19 and 20 are supported

by original claim 14, and in the specification at page 6, paragraph 0027. Finally, new claim 21 is supported by original claim 5.

Responsive to the Examiner's objection to the preliminary amendment filed July 20, 2001, a clean copy of new claims 17-21 is submitted herewith. Accordingly, entry of the present amendment is respectfully requested. Upon entry of this amendment, claims 17-21 are pending.

The Examiner's objection to claims 7-16 under 37 CFR 1.75 (c) regarding multiple dependent claims is rendered moot by the cancellation of these claims herein.

B. Rejections Under 35 U.S.C. § 102(b)

The rejection of claims 1-5 under 35 U.S.C. § 102(b) as allegedly being anticipated by Mottet, et. al. (*Virology*, 1996, Vol. 221, p.159-171), is rendered moot by the cancellation of these claims herein. In addition, it is respectfully submitted that the rejection does not apply to new claims 17-21.

Applicants' invention, as defined for example by claim 17, distinguishes over Mottet by requiring a method for transferring a foreign gene from a first cell to a second cell through contact infiltration, comprising inoculating a ribonucleoprotein comprising an RNA derived from a non-segmented (-)RNA virus into the first cell and allowing the first cell to contact a second cell, wherein the ribonucleoprotein has autonomous replication ability, and the RNA comprises a foreign gene, and lacks a gene encoding Matrix (M) protein or comprises an inactivated gene encoding M protein. For all of the reasons set forth below, Mottet does not describe such a method.

The ribonucleoproteins (comprising an RNA derived from a non-segmented (-)RNA virus) employed by the methods of the present invention have autonomous replication ability. In contrast, the viruses described by Mottet clearly do not have autonomous replication ability. In the experiments of Mottet *et al.*., RNA templates (such as E307 and CB119) are transcribed

under the presence of exogenous N, P, and L proteins or non-defective (ND) Sendai virus. In the *Materials and Methods* section of Mottet, is described the following:

> The cells were transfected with 2.5 μg of pGem4-N, 2.5 μg of pGem4-P/Cstop, 1 μg of pGem4-L, and 5 μg of the plasmid expressing the RNA template. (page 161, left column, second paragraph, emphasis added).

Method of preparing SeV mixed virus stocks of Mottet *et al.* is set forth below:

> This method which involves (i) the purification of the nucleocapsids..., (ii) their transfection in BHK cells infected with ND SeV and (iii) the further injection of these BHK cells into ... chicken eggs to produce the mixed virus stocks containing the ND and subgenomic RNAs... (third paragraph, emphasis added)

The subgenomic RNAs themselves cannot replicate autonomously. They replicate only "in a system where the viral functions L, P, and N needed for replication were also provided in trans" (page 162, left column, RESULTS, lines 5-7). In the absence of providing exogenous L protein, viral replication does not occur (see "-" lanes of FIG. 2, panel B, and FIG. 3, panel A of Mottet). In contrast, viruses of the present invention have autonomous replication ability. Exogenous N, P, and L proteins are required only in the first step for reconstituting the virus from cDNA. Once the virus is produced, it can autonomously replicate and contact infiltrate from a cell to another cell. Furthermore, since the subgenomic RNAs of Mottet lack any viral genes required for formation of an infective complex, they cannot infect other cells. They can form viral particles and infect other cells only in a mixture with ND viruses which provide functional viral proteins *in trans*.

In view of the mixture, the subgenomic RNAs of Mottet can replicate with the help of the ND virus. However, such a mixture can be distinguished from the virus of the present invention because the mixture contains the ND virus which has wild-type M protein. Finally, Mottet does not describe or suggest the ability of contact infiltration of a virus vector.

Clearly, Mottet does not describe each and every element of the methods required by present claims. Accordingly, it is respectfully submitted that the rejection under 35 U.S.C. § 102(b) does not apply to present claims 17-21.

The rejection of claims 1-6 under 35 U.S.C. § 102(b) as allegedly being anticipated by Magai, et. al. (EP 0 864 645), is rendered moot by the cancellation of these claims herein. In addition, it is respectfully submitted that the rejection does not apply to new claims 17-21. Applicants' invention, as defined for example by claim 17, distinguishes over Magai by requiring a method for transferring a foreign gene from a first cell to a second cell through contact infiltration, comprising inoculating a ribonucleoprotein comprising an RNA derived from a non-segmented (-)RNA virus into the first cell and allowing the first cell to contact a second cell, wherein the ribonucleoprotein has autonomous replication ability, and the RNA comprises a foreign gene, and lacks a gene encoding Matrix (M) protein or comprises an inactivated gene encoding M protein. Magai does not describe such a method.. Instead, Magai describes methods employing molecules that are clearly not capable of contact infiltration. Thus, Magai does not describe each and every element of the present invention. Accordingly, it is submitted that the rejection does not apply to new claims 17-21.

C. Rejection Under the Judicially Created doctrine of Obviousness-type Double Patenting

The rejection of claims 1-6 under the judicially created doctrine of obviousness-type double patenting in view of claims 76, 79-82, and 84-88 of copending Application No. 09/070,938 is rendered moot by the cancellation of these claims herein. In addition, copending Application No. 09/070,938 does not disclose or suggest methods for transferring a foreign gene from a first cell to a second cell through contact infiltration. Thus, it is respectfully submitted that the rejection does not apply to new claims 17-21.

D. Rejection under 35 U.S.C. § 103(a)

The rejection of claims 1-6 under 35 U.S.C. § 103(a) as allegedly being obvious over Nagai, et. al. (U.S. Application No. 09/070,938) is rendered moot by the cancellation of these claims herein. In addition, it is respectfully submitted that the rejection does not apply to new claims 17-21. Applicants' invention, as defined for example by claim 17, distinguishes over Nagai by requiring a method for transferring a foreign gene from a first cell to a second cell through contact infiltration, comprising inoculating a ribonucleoprotein comprising an RNA derived from a non-segmented (-)RNA virus into the first cell and allowing the first cell to contact a second cell, wherein the ribonucleoprotein has autonomous replication ability, and the RNA comprises a foreign gene, and lacks a gene encoding Matrix (M) protein or comprises an inactivated gene encoding M protein. Nagai does not disclose or suggest methods for transferring a foreign gene from a first cell to a second cell through contact infiltration. Thus, it is submitted that the rejection does not apply to new claims 17-21.

E. Rejection Under 35 U.S.C. 112, First Paragraph (written description)

The rejection of claims 1,2 and 6 under 35 U.S.C. 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventors, at the time the application was filed, had possession of the claimed invention, is rendered moot by the cancellation of these claims herein. New claims 17-21 are drawn to methods employing (-) strand RNA viruses. As acknowledged by the Examiner, Applicants provide a written description for methods employing(-) strand RNA viruses. Thus, it is respectfully submitted that the rejection does not apply to new claims 17-21.

F. Rejection Under 35 U.S.C. 112, Second Paragraph

The rejection of claims 1-6 under 35 U.S.C. 112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention, is rendered moot by the cancellation of these claims herein. With specific reference to the term "complex" and the phrases "genes encoding a protein" and

"the envelope and the virus core", this language does not appear in new claims 17-21, and therefore the rejection does not apply.

With specific reference to the phrase "derived from", as used in new claims 17 and 18, Applicants respectfully disagree with the Examiner's assertion that this phrase is allegedly indefinite. The phrase "derived from"is clearly defined in the specification (see specification, page 4, paragraphs 0018 and 0019). Accordingly, those skilled in the art would readily be able to determine the metes and bounds of claims 17 and 18.

With respect to the phrase "comprises no or inactivated gene encoding M protein", Applicants submit that this issue is rendered moot by the amended claim language used in the new claims. The Examiner's suggestion of acceptable alternative claim language is acknowledged with appreciation.

For all of the reasons set forth above, it is respectfully submitted that the rejection under 35 U.S.C. § 112, second paragraph does not apply to new claims 17-21.

CONCLUSION

In view of the above amendments and remarks, reconsideration and favorable action on all claims are respectfully requested. In the event any matters remain to be resolved, the Examiner is requested to contact the undersigned at the telephone number given below so that a prompt disposition of this application can be achieved.

Respectfully submitted,

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Enclosures: Exhibits A and B

DESCRIPTION

RNA VIRUS VECTOR WITH CONTACT INFILTRATION CAPABILITY

[0001] This application is a national stage application under 35 U.S.C. 371 of International Application (PCT) No. PCT/JP99/04333, filed on August 10, 1999, which claims priority from Japanese Patent Application No. 10/227398, filed August 11, 1998.

Technical Field

[0002] The present invention relates to a virus vector that can be used for gene therapy, and more specifically, to an inactivated negative strand RNA ((-)RNA) viral vector.

Background Art

[0003] In gene therapy for humans and animals, effectiveness and safety are very important. Particularly, in therapy using a "virus vector" which is obtained by viral gene recombination, treatment must be carried out with extreme care, since it is hard to discriminate between the possibilities of nonspecific chromosomal integration, leakage of recombinant or pathogenic viruses to nature, and uncontrolled expression of an introduced gene, even if the treatment is effective.

[0004] Virus vectors are mainly used for introducing a gene of interest into target cells by utilizing viral infectivity. Recombinant virus vectors, which have been genetically engineered to carry a gene insert(s), contain viral envelope proteins on the surface. Since the envelope proteins retain viral infectivity, it is possible to introduce its endogeneous recombinant gene inserts into cells. These vectors can be utilized for not only gene therapy but also production of cells expressing a gene of interest and creation of transgenic animals.

[0005] Virus vectors are classified into three categories: retrovirus vectors, DNA virus vectors, and RNA virus vectors. Among them, RNA virus vectors, which are not integrated into chromosomes, are advantageous in terms of safety. According to this technical background, virus vectors derived from a non-segmented (-)RNA virus such as the Sendai virus have already been provided (D. Yu et al., Genes to Cells, 2:457-466, 1997; M. Hasan et al., J. Gen. Virol. 78:2813-2820, 1997). The present inventors have developed a (-)RNA virus vector that has infectivity and autonomous RNA replication ability without disseminative capability (WO 97/16538).

Disclosure of the Invention

[0006] An objective of the present invention is to provide an RNA virus vector that can be used for gene therapy.

[0007] Using the nucleic acid of the Sendai virus[,] (a representative (-)RNA viruses), which is supposed to be the most useful vector for industrial applications in terms of safety and convenience, the present inventors succeeded in obtaining various defective mutants, which were experimentally reconstituted using known methods. As a result, the inventors found that the complexes reconstituted by the mutants carrying a defect in the M gene were capable of forming plaques, which were smaller than those formed by wild type viruses, but incapable of propagating in embryonated chicken eggs. Thus, the inventors confirmed that the mutants are capable of cell infection, autonomous RNA replication, and contact infiltration, but incapable of dissemination. Furthermore, the plaques formed by a defective mutant carrying a defect in the M gene were stained with the anti-Sendai virus antibody, but not with the anti-M monoclonal antibody. From this result the inventors confirmed that plaques formed by the M mutants are lacking complete M protein, thereby accomplishing the present invention.

[0008] Thus, the present invention provides RNA comprising genes involved in contact infiltration and autonomous RNA replication, but no genes or inactivated genes involved in dissemination.

[0009] The present invention also provides a cell comprising the above-described RNA, which is capable of allowing the RNA to replicate therein and transmitting [said] the RNA to another cell through contact infiltration.

[00010] Furthermore, the present invention provides a complex capable of cell infection, contact infiltration, and autonomous RNA replication but incapable of dissemination, a method for producing the complex, and a kit and host for producing the complex.

[0010] Moreover, the present invention provides a DNA comprising a template DNA for transcribing the above-described RNA *in vitro* or in a cell.

[0011] Herein, "cell infectivity" of a virus vector means "an ability of a viral vector capable of cell adhesion and membrane fusion to introduce its endogeneous nucleic acids into cells." "Disseminative capability" means "an ability of a nucleic acid introduced into a cell artificially or by infection to form infective particles or comparable complexes following replication of said nucleic acid in the cell, and then be transmitted into another cell." "Contact infiltration capability" means "an ability of a cell carrying a viral vector gene to transfer the gene to another cell through contact."

[0012] A viral vector capable of contact infiltration but incapable of dissemination can overcome a critical demerit of existing virus vectors, which are incapable of dissemination and contact infiltration. In other words, when cells are infected with a virus vector to express a gene insert in the virus vector, the existing virus vectors are only able to express a protein in

cells directly infected. This is not a problem when cell culture systems are used, in which the cells grow in a layer and can be directly infected with viral vectors. However, when *in vivo* cells that exist three-dimensionally in multi-layers are infected with viral vectors, the region and the number of cells directly infected by the existing vectors are limited. For example, it is extremely difficult to directly infect all tumor-forming cells at a tumor site with the viral vectors.

[0013] By contrast, even if virus vectors capable of contact infiltration of the present invention [is] are used to infect tumor cells at a tumor site and only a part of the cells are directly infected, noninfected cells adjacent to the infected cells can be infected with the vectors through contact infiltration, resulting in [infecting] infection of all the tumor cells. Since the vector is not disseminative, there is no possibility that the vector infects other cells in the body through the blood stream.

[0014] Thus, the virus vector of the present invention has such novel features that it infects all the cells in a restricted region or a particular organ and does not infect other parts of the body.

[0015] Furthermore, cells carrying the virus vector of the present invention capable of cell infection, autonomous RNA replication, and contact infiltration, but incapable of dissemination, can be utilized for cell transplantation. Since a cell carrying a virus vector capable of autonomous replication and contact infiltration can transfer the vector to another cell, it is possible to transplant the cell carrying the vector at the minimal dose to a diseased site[, thereby treating there].

[0016] It has been reported that the formation of infective particles of the Measles virus having a defect in the M gene is inhibited, and cell fusion is promoted (T. Cathomen et al., EMBO J., 17:3899-3908, 1998). However, the possibility to utilize the virus as a vector has not been suggested.

[0017] A source of a (-)RNA virus of the present invention includes the Sendai virus, the Newcastle disease virus, the Mumps virus, the Measles virus, the Respiratory syncytial virus, the rinderpest virus, and the distemper virus of the Paramyxoviridae; the influenza virus of the Orthomyxoviridae; and the Vesicular S[,] and Rabies viruses of the rhabdoviridae. Incomplete viruses such as DI particle (J. Virol. 68:8413-8417, 1994) and synthetic oligonucleotides can also be used.

[0018] A recombinant (-)RNA virus derived from any viruses described above can also be used as a vector source. For example, the recombinant (-)RNA viruses may be those with the gene for antigenicity inactivated or those with some genes altered to improve efficiency of RNA transcription or replication.

[0019] An RNA of the present invention can be obtained by transcribing an altered cDNA derived from any viruses or recombinant viruses described above *in vitro* or in cells. In the obtained RNA, at least one gene involved in disseminative capability of the original virus must be deleted or inactivated, but a gene involved in autonomous replication and contact infiltration should not be deleted or inactivated. In addition, an RNA molecule with artificial sequences, which are prepared by transcribing [is] *in vitro* or in cells [a DNA obtained by inserting genes for autonomous replication into a cDNA containing the structure of both ends of a virus genome such as DI molecules] can also be used. These RNA molecules can be

encoded by a DNA obtained by inserting genes for autonomous replication into a cDNA containing the structure of both ends of a virus genome, such as DI molecules.

[0020] In the Sendai virus, "genes involved in autonomous replication" are either of NP, P/C, or L genes, and "gene[d]s involved in transmission" are either of M, F, or HN genes. When only the M gene is deleted or inactivated, "disseminative capability" will be lost, but "contact infiltration ability" will remain because virion or virus-like particles capable of releasing the complex containing genomic RNA extracellularly are not formed. Therefore, for example, an RNA of Sendai virus Z strain deficient in only the M gene and a ribonucleoprotein (RNP) comprising [said] the RNA are suitably used in the present invention. The genes [are] do not [necessary] necessarily have to be the same as the original viral sequences. [They] The genes can be mutated or substituted by the corresponding gene of other viruses as long as the resulting gene has transcription and replication activities comparable to or higher than those of the wild type gene.

[0021] An RNA of the present invention may have a foreign gene inserted into an appropriate site. To express a desired protein, a foreign gene encoding the protein is inserted. In the case of Sendai virus RNA (GenBank accession No. M30202), a sequence with a multiple of six bases between the R1 and R2 sequences (J. Virol., Vol. 67, No. 8, 1993, 4822-4830) is inserted. The consensus sequences of R1 and R2 are 5'-AGGGWBAAWGD-3' and 5'-DTAAGAAAAA-3', respectively. The expression level of the inserted foreign gene can be regulated by the inserted position or the RNA nucleotide sequences flanking the inserted gene. For example, [it is known about] for Sendai virus RNA, it is known that the expression level of the inserted gene is higher as the insert position is nearer to the NP gene.

[0022] A complex of the present invention comprises a virus structure without the above-described RNA and nucleic acid. "A virus structure without nucleic acid" is, for example, a virus from which only RNA is removed and complements infectivity and autonomous replication ability of the RNA of the present invention[,]. However, "a virus structure without nucleic acid" does not complement the ability to form particles that release the complex extracellularly and move freely, in other words, does not complement the disseminative capability. In the Sendai virus, a complex of the RNA in which only the M gene is deleted and the virus structure from which the RNA and M protein are removed (RNP), has infectivity and autonomous replication ability, but not disseminative capability. The complex can contain any other components as long as they do not exhibit disseminative capability. For example, molecules that bind[s] to a specific cell, such as adhesion molecules, ligands, or receptors, can be included on the envelope surface.

[0023] Moreover, the present invention relates to a kit comprising a) the above-described RNA of the present invention, a cRNA of [said] the RNA, or a unit that is capable of biosynthesizing [said] the RNA or [said] the cRNA, and b) a group of enzymes required for replication of [said] the RNA or [said] the cRNA, or a unit that is capable of biosynthesizing [said] the enzymes. The complex of the present invention, which is capable of cell infection, autonomous RNA replication, and contact infiltration, but incabable of dissemination, can be produced by introducing units of a) and b) into an appropriate host. Producing the complex of the present invention means reconstituting an RNP having autonomous replication ability. When RNA derived from the Sendai virus is used for a), b) is preferably all of NP, P/C, and L proteins derived from the Sendai virus or a unit capable of biosynthesizing [said] the proteins.

[0024] [A host is not limited as long as it] Hosts contemplated for use in the practice of the invention can be any host that allows virus RNA to be expressed therein. When RNA derived from the Sendai virus is used, any cells susceptible to the infection with Sendai virus can be used, including cultured mammalian cells, cultured avian cells, and embryonated chicken eggs. Examples of cultured cells are LLCMK2, MDCK, MDBK, CV-1, Hela, HepG2, P19, F9, CHO, PC12, 293 cell, BAF3, Jerkat, human PBMC, MT-4, Molt-4, NIH3T3, L929, chicken embryo fibroblast, and the like.

[0025] The present inventors have shown that Sendai virus cDNA reconstitutes particles more efficiently when cDNA [to be] is introduced into cells [is] in a linear form rather than in a circular form, and that transcription of a (+)RNA in cells more successfully forms particles than that of a (-)RNA (A. Kato et al., Genes to Cells, 1: 569-579, 1996). Although these conditions may not always be applicable to the reconstitution of all other (-)RNA viruses, it is possible to determine the appropriate conditions for the reconstitution of other (-)RNA viruses according to the description of the present specification and based on conventional technology.

[0026] The produced complex can be recovered by [the] standard methods from the host, for example, cultured cells or embryonated chicken eggs.

[0027] A foreign gene encoded by the RNA constituting a complex can be expressed by infecting cells with the complex. Namely, the foreign gene can be expressed *in situ* by inoculating the complex or a cell carrying it into a host such as embryonated chicken eggs or appropriate tissues of an animal body. Alternatively, cells recovered from a living body are transformed so that the complex can autonomously replicate, and the transformed cells are returned to the living body to allow the cells to express the foreign gene.

[0028] As an embodiment of the present invention, Figure 9 shows the processes of infecting target cells with a Sendai virus complex in which the M gene is deleted or inactivated and infiltrating the RNA of the present invention into surrounding non-infected cells through contact infiltration.

Brief Description of the Drawings

[0029] Figure 1 schematically shows the structure of the plasmid pUC18/T7(+)HVJRz.

[0030] Figure 2 schematically shows the region carrying the M gene in Sendai virus cDNA and procedure for subcloning or mutating the region. In the figure, E stands for transcription termination sequence; I, intervening sequence; and S, transcription initiation sequence. Boxes indicate the restriction sites used for the plasmid construction.

[0031] Figure 3 schematically shows procedures for introducing mutation to the M gene region of Sendai virus cDNA. The upper panel of A shows the structure of the Sendai virus genome with each position of the NP, P(P/C), M, F, HN, and L genes. Lower panel shows the structure of the M gene, restriction sites, and positions of primers M1 and M2 used for the construction of the M defect type. B and C schematically show the construction of the M defect type and the M deletion type, respectively. Crossed out restriction sites indicate that they are mutated to be indigestible by the enzymes. D shows a comparison between the M defect and wild type M genes in terms of both nucleotide and amino acid sequences. Dots in the M defect sequences mean that bases or amino acids are identical to the corresponding wild type sequences. "Ter" indicates a termination codon. Numerals indicate the base/amino acid position in the M gene sequence.

[0032] Figure 4 schematically shows a procedure for subcloning the M gene region of Sendai virus cDNA. Crossed out restriction sites indicate that they are mutated to be indigestible by the enzymes.

[0033] Figure 5 schematically shows the construction of the M deletion type cDNA by mutating the subcloned Sendai virus M gene region and replacing the full-length M gene region of Sendai virus cDNA with the mutated one. Crossed out restriction sites indicate that they are mutated to be indigestible by the enzymes.

[0034] Figure 6 schematically shows the construction of the M defect type cDNA by mutating the subcloned Sendai virus M gene region, and replacing the full-length M gene region of Sendai virus cDNA with the mutated one. Crossed out restriction sites indicate that they are mutated to be indigestible by the enzymes.

[0035] Figure 7 shows plaques of Sendai virus cDNA of wild type (WT1 and WT2); M defect type (dM); and M deletion type (dMEA2-1 and dMEA2-2).

[0036] Figure 8 shows plaques of Sendai virus cDNA of wild type (WT); M defect type (dM); and M deletion type (dMEA), which were stained with anti-Sendai virus antibody.

[0037] Figure 9 illustrates the process of contact infiltration by Sendai virus genome of wild type genome (upper panel) and of the mutant with mutated or no M gene (lower panel). The wild type forms infective virus particles and causes contact infiltration, while the mutant is not able to form infective particles but introduces its genome into non-infected surrounding cells only by contact infiltration.

Best Mode for Carrying out the Invention

[0038] The present invention will be illustrated in detail below with reference to the following Examples, but is not to be construed as being limited thereto.

Example 1. Subcloning of the M gene of Sendai virus

[0039] All of the following ligation, blunting, and dephosphorylation were performed using the Takara Ligation Kit Ver.2 (Takara Shuzo, Kyoto, Japan), the Takara Blunting Kit (Takara Shuzo), and CIAP (Takara Shuzo), respectively, according to the manufacture's protocols attached to the products. The nucleotide sequences were determined by Sanger's method (F. Sanger, Science, 214: 1205-1210, 1981).

[0040] Complementary DNA in which the M gene was deleted was constructed as follows, using wild type cDNA, pUC18/T7(+)HVJRz (A. Kato et al., Genes to Cells, 1: 569-579, 1996) (Figure 1). The DNA sequences of the M gene and its vicinity are shown in Figure 2, and the construction scheme is shown in Figure 3.

[0041] First, the ClaI fragment containing the M gene was excised from pUC18/T7(+)HVJRz and inserted into the ClaI site of pHSG396 (Takara Shuzo) to obtain pHSG-M-CC (Figure 4). Separately, pHSG396 was digested with ApaLI, blunted, and self-ligated to obtain pHSG396-dA that lacks the ApaLI site (Figure 4). The EcoRI-BamHI fragment of pHSG-M-CC carrying the M gene was inserted into dephosphorylated EcoRI-BamHI-digested pHSG396-dA to obtain pHSG-dA-M-BE (Figure 4).

Example 2. Construction of M deletion type vector

[0042] Forty nine-mer synthetic oligonucleotides, 5'-ggcgatatctatagattccctaagttctcatagtagatgtgcaccggca-3' (EA linker F) (SEQ ID NO: 1) and 5'-tgccggtgcacatctactatgagaacttagggaatctatagatatcgcc-3' (EA linker R)(SEQ ID NO: 2), were

annealed and inserted into pCR2.1 (Invitrogen) to generate pCR-EAL. The nucleotide sequence of the insert was verified to be as designed.

[0043] The pCR-EAL was digested with EcoRV and ApaLI, and the linker fragment was excised. Separately, the pHSG-dA-M-BE was digested with EcoRV and ApaLI and the above linker fragment was inserted therein to obtain pHSG-dA-dM-EA-EB.

[0044] The pHSG396 was digested with EcoRI, blunted, and self-ligated to obtain a plasmid lacking the EcoRI site, which was further digested with BamHI, blunted, and self-ligated to obtain pHSG-dE-dB, a plasmid lacking both EcoRI and BamHI sites.

[0045] The ClaI fragment carrying the M gene was inserted into the ClaI site of pHSG-dE-dB to generate pHSG-dE-dB-M-CC. The EcoRI-BamHI fragment excised from pHSG-dA-dM-EA-EB was inserted into the EcoRI-BamHI-digested pHSG-dE-dB-M-CC to obtain pHSG-dE-dB-dM-CC.

[0046] The ClaI fragment of pHSG-dE-dB-dM-CC was inserted into the ClaI site of the pUC18/T7(+)HVJRz to obtain pHVJ-dMEA. This construction scheme is shown in Figure 5.

Example 3. Construction of M defect type vector

[0047] PCR was performed using pUC18/T7(+)HVJRz as a template with oligonucleotide primers, M-1 (5'-ttaaaggcctaaaccgatctcagaattacg-3')(SEQ ID NO: 3), and M-2 (5'-tatcattccctgtctcagcctgcc-3')(SEQ ID NO: 4). The primers were designed to introduce a mutation at the BsgI site of the M gene so that a stop codon appears in frame of the M gene. The PCR products were subcloned into pCR2.1 to obtain pCR-M (Figure 6).

[0048] Following verification of the nucleotide sequence of pCR-M, the StuI-XcmI fragment of pCR-M was recovered from the gel and inserted into the StuI-XcmI-cut pHSG-M-CC. The resulting plasmid was named as pHSG-dM-CC. The ClaI fragment of pHSG-dM-CC was inserted into the ClaI site of pUC18/T7(+)HVJRz to obtain pHVJ-dM. This construction scheme is shown in Figure 6.

Example 4. Reconstitution of virions from M deletion type and M defect type cDNAs

[0049] Reconstitution of virions from cDNA of wild type (WT), M deletion type (dMEA), and M defect type (dM) was performed as follows.

[0050] Cultured LLCMK2 cells (a simian kidney cell line) were detached from a dish by treatment with trypsin, and 2 x 10^6 cells were plated in a plastic dish of 10 cm diameter and incubated in MEM medium (Gibco BRL) supplemented with 10% FBS (Gibco BRL) at 37°C for 24 hours in 5% CO_2. The medium was then removed, and the cells were washed once with PBS. Five hundred µl of a solution of vTF7-3, a recombinant vaccinia virus vector capable of expressing T7 phage RNA polymerase (T.R. Fuerst et al., Proc. Natl. Acad. Sci. USA, 83:8122-8126, 1986), which was diluted with PBS to 8 x 10^6 pfu/ml to obtain MOI (multiplicity of infection) of 2, was added dropwise to the dish. The cells were infected for 1 hour with agitating the dish every 15 minutes to spread the virus solution over the whole dish.

[0051] Separately, a medium containing a cDNA solution was prepared as follows. First, 500 µl Opti-MEM (Gibco BRL) without FCS (fetal calf serum) was added into a polystyrene tube, and then any of pUC18/T7(+)HVJRz, pHVJ-dMEA or pHVJ-dM, all plasmids originating from Sendai virus cDNA (20 µg), pGEM-NP (5 µg), pGEM-P (2.5 µg), and pGEM-L (5 µg) were added. Fifty µl SuperFect (QIAGEN) was further added, and the mixture was allowed to stand at room temperature for 20 minutes. Ten ml of Opti-MEM

was then added. Subsequently, Rifampicin and Cytosine arabinoside (Ara C) were added at the final concentration of 100 μg/ml and 40 μg/ml, respectively.

[0052] After 1-hour infection, the virus solution was removed from the above dish containing LLCMK2 cells, the medium containing the above cDNA solution was added to the culture by decantation, and the cells were incubated at 37°C for 48 hours in 5% CO_2. The cells were scraped using a cell scraper without removing the medium, and the cells and the medium were transferred together to a 15-ml centrifuge tube. After centrifugation at 1200 rpm for 5 minutes, the supernatant was removed, and the precipitated cell was resuspended in 200 μl of PBS.

Example 5. Inoculation of cell suspensions to embryonated chicken eggs and HA assay

[0053] To evaluate virus reconstitution, an assay using embryonated chicken eggs was performed. The cells suspended in 200 μl of PBS obtained in Example 4 were adjusted to 1 x 10^7 cells/ml and sequentially diluted with PBS to obtain cell suspensions of 1 x 10^6 and 1 x 10^5 cells/ml. One hundred μl of each suspension was inoculated to a 10-day-old embryonated chicken egg through the air chamber side using a 24G needle.

[0054] The chicken eggs were incubated at 35.5 °C for 3 days with rotating. Then, the eggshell at the air chamber was cut out, and urine was collected using a 10-ml syringe with 18G needle.

[0055] The collected urine was tested by HA assay to evaluate virus propagation in the chicken eggs. HA assay was performed as follows.

[0056] Fifty μl of PBS was dispensed into each well of from the second to the 12th lines of a 96-well round bottom plate. One hundred μl of the urine sample was added to the first line. Fifty μl of the sample in the first line was mixed with PBS in the second line to make a 1:2 dilution. Fifty μl of the 1:2 dilution was mixed with PBS in the third line to make a 1:4 dilution. By repeating the procedure, a series of two-fold dilutions were made.

[0057] Fifty μl of 1% chicken preserved blood (Cosmo Bio) diluted with PBS was added to all the lines, and the plate was kept at 4°C for 1 hour. Erythrocyte aggregation was examined with the naked eye and the highest dilution that results in the aggregation was shown as HA activity (Table 1).

Table 1

Virus cDNA	HA activity
WT (wild type)	> 16
dM (defect)	< 2
dMEA2-1 (deletion)	< 2
dMEA2-2 (deletion)	< 2

[0058] Virus cDNAs used were of viruses, wild type (pUC18/T7(+)HVJRz; WT), the M defect type (pHVJ-dM; dM), and the M deletion type (pHVJ-dMEA; dMEA). For the M deletion type, the results of two experiments were shown (dMEA2-1, and dMEA2-2). The wild type virus showed an activity higher than 16, whereas the other samples did not show any significant activity. The result indicates that the Sendai virus cDNA in which the M gene is deleted or is defective did not produce a virus with disseminative capability in the standard reconstitution experiment.

Example 6. Plaque formation

[0059] CV-1 cells were plated at 5 x 10^5 cells/well into a 6-well plate (Corning, microplate 6-well) and cultured overnight.

[0060] LLCMK2 cells obtained in Example 4 were diluted with PBS to 1 x 10^6 cells/ml, and 100 µl of the suspension was dispensed into 1.5 ml microtubes (Eppendorf). A portion of the dispensed suspensions was subjected to three repeats of freeze-thawing by freezing at –80°C for 10 minutes and thawing in water at room temperature for 3 minutes. Trypsin was added to all the cell suspensions at the final concentration of 0.75 µg/ml and the mixtures were incubated at 37°C for 30 minutes.

[0061] The media were removed from the plates of CV-1 cell culture, and the cells were washed once with PBS, then overlaid with 100 µl of the above diluted cell suspension treated with trypsin. The mixtures were incubated for 1 hour with agitating every 15 minutes so as to spread the suspension over the whole plate. The plates were overlaid with 3 ml of 1 x MEM containing 1% agar, supplemented with 0.1% BSA, Rifampicin 100 µg/ml, Ara C 40 µg/ml, and trypsin 0.75 µg/ml, that was preheated to approximately 45°C. After the agar solidified, the plates were inverted and incubated at 37°C for 5 days in 5% CO_2.

[0062] Then, 1 ml of a fixative (ethanol:acetic acid = 5:1) was added onto the agar, and the plates were allowed to stand at room temperature for 90 minutes. The agar was then removed, and 200 µl of Amido Black solution (0.5% Amido Black, ethanol:acetic acid:water = 45:10:45) was added. Immediately thereafter, the cells were washed with water and stained to count the plaque number and evaluate the shapes of plaques. The results were shown in Table 2 and Figure 7.

Table 2

Reconstitution efficiency of Sendai virus deficient or defective in the M gene

M gene	Number of plaques		
	freeze-thawed		not frozen
	Run 1	Run 2	
wild type	38	22	14
M defect type (dM)	2	4	2
M deletion type (dMEA2-1)	27	26	60
M deletion type (dMEA2-2)	32	38	42

[0063] Virus cDNAs used are of wild type (pUC18/T7(+)HVJRz), the M defect type (pHVJ-dM), and the M deletion type (pHVJ-dMEA). For the M deletion type, the results of two experiments (dMEA2-1 and dMEA2-2) are shown. The number of plaques was counted for both freeze-thawed and not frozen cell suspensions. The plaque number reflects the number of reconstituted viruses in 1 x 10^5 transfected LLCMK2 cells. Experiments using freeze-thawed samples were performed twice (runs 1 and 2). Although it was reported that reconstitution efficiency of the Sendai virus decreases in a mutant virus such as an addition type in which a foreign gene was inserted into genome (Hasan et al., J. Gen. Virol. 78: 2813-2820, 1997), the M deletion type showed a comparable reconstitution efficiency to that of wild type. The M defect type showed a little lower efficiency. Freeze-thawing of the transfected cells did not significantly affect the plaque number.

[0064] As is evident from Table 2 and Figure 7, plaques that are supposed to be derived from cDNAs of the M deletion type and the M defect type were observed. The size of the plaques was much smaller than that of wild type formed in simultaneous positive control (Figure 7; WT1 and WT2). To further examine the shape of the plaques, they were stained with anti-

Sendai virus polyclonal antibody, which was produced by the known method by inoculating rabbits with purified Sendai virus that was inactivated as antigens.

[0065] The plaques described in Example 6, which were fixed and stained with Amido Black, were hybridized with anti-Sendai virus antibody, and subsequently with FITC-conjugated anti-rabbit IgG or with anti-mouse IgG antibody, then observed under fluorescence stereoscopic microscope. As a result, signal was detected in plaques of wild type, the M deletion type, and the M defect type, confirming that the plaques were of Sendai viruses (Figure 8).

[0066] It was observed that the plaques of wild type cDNA were almost circular, whereas those of the M deletion type and the M defect type cDNAs were small and not circular, having irregular shapes (Figure 8).

Example 7. Antibody staining of plaques

[0067] To determine whether the plaques, different from those of wild type in size and shapes, are derived from cDNA of the M deletion type or the M defect type, the presence of M protein was examined. Namely, these plaques were stained with anti-M protein monoclonal antibody and observed under fluorescence stereoscopic microscope.

[0068] Immunofluorescence staining of the plaques was performed as follows. Plaques were stained with Amido Black. The position of the plaques was examined with the naked eye and marked with a marker pen. Each well of 6-well plates that contains plaques was washed once with 1 ml of PBS, overlaid with 200 µl of 1:40 diluted anti-M protein monoclonal antibody, and incubated at 37°C for 45 minutes in a CO_2 incubator. Then, the well was washed three times with 1 ml of PBS, overlaid with 200 µl of 1:100 diluted FITC-conjugated anti-mouse

IgG antibody (Cosmo Bio), and incubated at 37°C for 45 minutes in an incubator. Finally, each well was washed three times with 1 ml of PBS, overlaid with 1 ml of PBS. The staining of the marked plaques was then examined under fluorescence stereoscopic microscope.

[0069] Thereafter, the same procedure was repeated processed using anti-Sendai virus polyclonal antibody as the first antibody and FITC-conjugated anti-rabbit IgG antibody (ICN Biomedicals) as the second antibody. The wells were then observed under fluorescence stereoscopic microscope again.

[0070] The plaques from wild type cDNA were stained with both anti-Sendai virus antibody and anti-M protein monoclonal antibody. In contrast, plaques from cDNA of the M deletion or defect type were stained with anti-Sendai virus antibody, but not with anti-M protein monoclonal antibody. This indicates that the latter plaques, lacking complete M protein, are derived from cDNA of the M deletion and defect types.

[0071] Accordingly, plaques from a Sendai virus gene having a deletion or defect in the M gene were obtained. Since the virus forming plaques does not propagate in chicken eggs, the virus is supposed to be incapable of budding, i.e. not disseminative. In addition, the virus is supposed to be capable of contact infiltration as it forms plaques.

[0072] This mode of propagation is probably due to cell fusion mediated by F and HN proteins. Namely, among proteins encoded by Sendai virus, F and HN proteins are expressed as a membrane protein on the cell surface, and M protein supports F and HN proteins as an intracellular anchor in virus budding. In case of the M deletion type and M defect type, budding does not occur because the anchor is lost. However, F and HN proteins are expressed on the cell surface, and cell fusion occurs mediated by these proteins, so that virus

genome can be transferred into surrounding cells. In this way, it is possible to realize virus propagation without budding (Figure 9).

Industrial Applicability

[0073] The present invention provides an RNA virus vector capable of cell infection, autonomous RNA replication, and infiltration through contact, but incapable of dissemination. By utilizing the RNA virus vector of the present invention, it is possible to carry out more efficient gene introduction and cell transplantation in gene therapy.

ABSTRACT

The present invention provides an RNA virus vector useful for gene therapy. The RNA virus vector is capable of cell infection, autonomous RNA replication, and infiltration through contact, but incapable of dissemination. The use of the RNA virus vector of the present invention provides more efficient gene transfer and cell transplantation in gene therapy compared with conventional techniques.